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Development of a Serodiagnostic Test to Determine Exposure of Tortoises to Herpesvirus

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An Enzyme Linked Immunosorbent Assay (ELISA) for Detecting Herpesvirus Exposure in Mediterranean Tortoises [Spur-Thighed tortoise (*Testudo graeca*) *and* Hermann's tortoise (*Testudo hermannii*)]

Summary

An enzyme-linked immunosorbent assay (ELISA) was developed for detection of antibodies to a herpesvirus associated with an upper respiratory tract disease in Mediterranean tortoises [spur-thighed tortoise (*Testudo graeca*) and Hermann's tortoise (*Testudo hermannii*)]. This serodiagnostic test was validated through an immunization study. The mean of the OD_{405} reading of the negative background plus three times the standard deviation was used as the cutoff for seropositive tortoises. ELISA results were compared to serum neutralization (SN) values for the same samples. The results obtained by SN and ELISA were not significantly different ($P > 0.05$). This new ELISA test could be used as an important diagnostic tool for screening wild populations, private, and zoo collections of Mediterranean tortoises.

Introduction

Herpesviruses are well known infectious agents with a remarkable wide host range. Starting in 1975 (Rebel et al.) several reports have documented the presence of herpesvirus-like particles in land tortoises, fresh water and marine turtles (Cox et al 1980; Drury et al 1998/1999; Frye et al., 1977; Harper et al., 1982; Heldstab and Bestetti, 1989; Jacobson et al., 1982/1985/1986/1991; Kabish and Frost 1994; Marschang et al., 1997/1998/1999; Muro et al., 1998; Une et al, 1999). More recently, an increasing number of investigations have revealed an association between the presence of herpesvirus and an upper respiratory disease in Mediterranean tortoises [spur-thighed tortoise (*Testudo graeca* and Hermann's tortoise (*T. hermannii*). (Drury et al 1998/1999; Heldstab and Bestetti, 1989; Jacobson et al.,1985; Kabish and Frost 1994; Marschang et al., 1997/1998/1999; Muro et al., 1998).

In tortoises with herpesvirus infection, clinical signs described in the literature range from a mild conjunctivitis to a severe stomatitis-glossitis and pharyngitis. Diphteritic plaques can be observed on the dorsal surface of the tongue and on the hard palate of infected tortoises. Frequently a clear serous to a muco-purulent nasal discharge is present. Signs of central nervous system disease have also been reported in Mediterranean tortoises with herpesvirus infection (Heldstab and Bestetti, 1989).

Using light microscopy eosinophilic intranuclear inclusions are often seen in multiple tissues. These are particularly prominent in those tortoises with pharyngitis and glossitis. By transmission electron microscopy (TEM) inclusions consist of numerous viral particles. The morphology and morphogenesis have been used to categorize the virus as herpesvirus.

A diagnosis of herpesvirus infection is often made solely based upon light or electron microscopy finding. Antemortem diagnosis can be made using biopsy specimens of oral lesions. A serum neutralization (SN) test has been developed but is limited in its application since it is only available in a few research laboratories in Europe (Frost and Schmidt, 1997). However, time is a limiting factor with the SN test. Ten to fourteen days are required to obtain the final reading and a laborious procedure is required. An easier and faster but equally reliable serodiagnostic test is needed. In this report we describe the development of an enzyme-linked immunosorbent assay (ELISA) that can be used to monitor the exposure to herpesvirus of free ranging, private and zoo collections of tortoises.

Material and Methods

Viruses

Herpesvirus isolates HV1976 and HV4295/7R/95 were used as antigens in the ELISAs and immunoblots. Both isolates were obtained from Hermann's

tortoises. HV1976 was isolated from a dead pet Herman's from the US (Washington State) while HV4295/7R/95 was isolated from a pet Herman's tortoise in Germany during an herpesvirus outbreak in a private collection (Marschang et al., 1997).

Antigen Preparation for ELISA

For use as ELISA antigens, the herpesvirus isolates were grown in TH-1 cell monolayers (Terrapene Heart cells, ATCC-CCL 50 Sub-line B1, Rockville, MD), in T-150 plastic flasks with ventilated cap (Corning, New York). TH-1 cells were grown in DMEM/F12 medium (GibcoBRL, Grand Island, NY) with 5% fetal bovine serum (FBS) (Sigma, St Louis MO), Gentamycin (30 mg/500ml) (Sigma, St Louis MO) and with an antimycotic-antibiotic solution (ABAM, Sigma, St Louis, MO) containing Penicillin G (60,000 U/500ml), Streptomycin (60,000U/500ml), and Amphotericin B (150 µg/500ml). Infected cell monolayers were scraped and collected with the culture medium, at the highest point of the virus growth curve. The cell suspension was then frozen at -80 °C and thawed three times and the supernatant was clarified at 4,500X g for 30 min at 4 °C in 50ml centrifuge tubes using a refrigerated centrifuge (Sorvall RC3, Norwalk, CT). The pelleted cell debris was discarded and the clarified supernatant was ultracentrifuged at 53,664X g at 4°C for 3.5 hours to pellet the virus in 50 ml ultracentrifuge tubes (Beckman, Palo Alto, CA) using a T-21 Beckman rotor in a ultracentrifuge (Beckman L-55). The resuspended pellets were purified on 20-60% sucrose

continuous gradients in TNE (Tris, NaCl, EDTA) (pH 7.5), in 14x89 mm polyallomer ultracentrifuge tubes (Beckman, Palo Alto, CA), and ultracentrifuged at 156,194X g for 2 hours at 4°C, using a Beckman SW41 rotor. A total of 9 fractions of about 1 ml each were harvested. The amount and the quality of the virus contained in each of the fractions was assessed with: 1) a protein assay test (Bio-Rad, Hercules, CA); 2) by evaluating cytopathic effect (CPE) in TH-1 cells and determining titers in 96 well flat bottom plates (according Spearman and Karber – Hierholzer and Killington, 1996); and 3) using negative staining electron microscopy (NEM). The fractions richest in the purest virus (assessed as described above) were used for the production of two rabbit polyclonal antibodies (raised against HV4295/7R/95 and HV1976 respectively) and for use in an immunization study. The antigen used in the ELISA was selected from the gradient fractions richest in virus. These fractions were resuspended in 10 volumes of TNE (pH 7.5) and re-pelleted by ultracentrifugation at 53,664X g for 3.5 hours at 4°C. The pellet was then resuspended in phosphate buffered saline (PBS) (pH 7.2) and stored at –80 °C.

Antigen Preparation for Immunoblotting

Th-1 cells infected with either HV4295/7R/95 or HV1976 and uninfected TH-1 cells were used for immunoblotting. Infected cells were harvested when they showed 80-90% CPE, while uninfected cells were harvested at confluency. After discarding the media, the cell monolayer was washed three times with PBS (pH

7.2) and cells were scraped. Cells were then harvested and centrifugated at 250X g and resuspended in 0.9 ml of PBS. After the cells had been frozen and thawed three times, 0.1 ml of a lysis buffer (0.4% NP40, 0.4% Tween 20, 01% SDS in DDH_20) was added to the suspension reaching a final volume of 1 ml. The suspensions were incubated at room temperature for 1 hour and the final protein concentration was determined with a protein assay test (Bio-Rad, Hercules, CA), following the instructions of the manufacturer. The antigen preparations were stored at –80 °C.

Tortoise Plasma Samples

Blood samples were collected from either the dorsal tail vein or the jugular vein of each tortoise. Blood was placed in lithium-heparinized plastic tubes (Becton Dickinson, Rutherford NY) to prevent clotting. Plasma was obtained by low speed centrifugation of the tubes at 350X g in a TRIAC centrifuge (Clay Adams, Becton Dickinson and Company, Parsipanny New Jersey) for 5 min at room temperature. All the plasma samples were stored at –80 °C until the analyses were performed.

Samples from Mediterranean tortoises in France. The plasma samples from France were collected from a group of 175 captive Mediterranean tortoises. All samples were previously tested by SN using three herpesvirus isolates isolated from Mediterranean tortoises (HV770, HV2245, HV17/96; Mathes, pers. comm.)

in Europe. The tortoises were considered seropositive when their sera successfully neutralized at least one of the herpesvirus isolates. The tortoises were considered seronegative when no neutralization activity was detected against any of the isolates used in the test. In addition, serial plasma samples were collected from five spur-thighed tortoises used in the immunization study described below.

Samples from immunized tortoises. Five adult male spur-thighed tortoises SN negative for exposure to tortoise herpesvirus and culture negative for tortoise herpesvirus were purchased from a local reptile dealer and used in an immunization study. Seven days prior to immunization the tortoises were separated into individual pens. The tortoises were divided in three treatment groups: 1) Group 1 - two tortoises immunized with HV4295/7R/95 (European isolate); 2) Group 2 - two tortoises immunized with HV1976 (American isolate); and 3) Group 3 - one control tortoise. For each immunization group, live virus was delivered either intramusculary (IM; tortoises #3 and #4) or intranasally (IN; tortoises #1 and #2) with the dose of 15,000 TCID50 re-suspended in 0.4 ml of PBS pH 7.2 (purified virus batches from HV4295/7R/95 passages 19-26 and HV1976 passages 14-15). The control tortoise (#5) received 0.4 ml of PBS pH 7.2 both IN and IM. The tortoises were bled immediately before the virus administration (time 0) and every two weeks for a total of 17 and 15 weeks respectively for the tortoises injected intranasally (#1 and #2) and IM (#3 and #4). The tortoises were hibernated in the winter for 6 weeks. Following hibernation,

the tortoises were bled every 4-5 weeks. Plasma was separated by centrifugation and tested for the presence of serum-neutralizing antibodies and with the ELISA assay.

Virus isolation was attempted from each tortoise in the immunization study. Briefly, starting seven days after immunization and every two weeks thereafter until the hibernation period (17/15 weeks, after IN/IM immunization respectively), viral isolation was attempted from nasal flushes and pharyngeal swabs. One milliliter of 0.9% sterile saline was alternatively flushed and aspirated repeatedly into both nares of each tortoise. A DMEM/F12 wetted dacron tip sterile swab (Hardwood Product Company, Guilford, Maine) was used to collect a sample from the mucosal surface of the pharynx. After collection, the samples were stored in 15ml centrifuge tubes containing DMEM/F12 cell culture medium with the appropriate antimicrobials. The tubes were kept on ice until delivered to the laboratory. Subsequently, the transport media was filtered with 0.45 μm syringe filters (Fisher Scientific, Pittsburgh PA) and added to fresh TH-1 cell confluent monolayers in $25cm^2$ cell culture flasks (Corning, N.Y.) and incubated at 28°C. After 1 hour the media was discarded and fresh media added with 5% FBS and incubated in the same conditions described above. Cells were monitored daily for CPE. One blind passage was performed after seven days. No CPE detection after two weeks was considered a negative result.

Hybridoma Preparation

Mouse monoclonal antibodies (Mab) against Mediterranean tortoise immunoglobulin (IgY) heavy chain (Hadge et al., 1980; Leslie et al., 1969; Warr et al., 1995) were produced by following a standard hybridoma protocol in the University of Florida Hybridoma Core laboratory (Kao et al., 1986; Liddell et al., 1991; Simrell et al, 1979). IgY was purified from Mediterranean tortoise plasma (Andreas and Ambrosius, 1989) and was used to immunize 6- to 8 week-old female BALB/C mice. The spleen cells of the hyperimmune mouse were fused with myeloma cells (Sp2/O). Supernatants from the resulting hybridoma cells were screened by ELISA and Western blotting for antibody specific for Mediterranean tortoise IgY Heavy chain [IgY (H)]. The Mab specific for Mediterranean tortoise IgY(H) was designated HL1546. Mab HL1546 was isotyped as an immunoglobulin G1 (IgG1) by using a mouse Mab isotyping kit (Stratagene Iso Detect mouse isotyping kit, La Jolla, CA). The HL1546 MAb was purified on protein G affinity column (Pharmacia Hi-Trap Protein G Sepharose High Performance; Pharmacia LKB, Uppsala, Sweden) and biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL)(Goding, 1986).

ELISA Procedure.

For the ELISA procedure a modified protocol developed at the University of Florida for detecting mycoplasma exposure in tortoises, was adopted

(Schumacher et al., 1993). Briefly, each well of a microtiter plate (Maxi-sorp F96; Nunc, Kamstrup, Denmark) was coated with 50 µl of antigen at a concentration of 5µg/ml in 0.01M sodium phoshate buffer (pH 7.2) containing 0.15 M NaCl and 0.02% NaN_3 (PBS/A) and the plates were incubated at 4°C overnight (Harbeck and Giclas, 1991). The wells were washed four times with PBS/A containing 0.05% Tween 20 (PBS-T) with an automatic ELISA washer (EAW II; LT Labinstruments, 5082 Groedig/ Salzburg, Austria) and then blocked with 300 µl of PBS/A containing 5% nonfat dry milk per well at room temperature for 60 min or at 4°C ovenight. After four more washes, 50 µl of plasma diluted 1:25 in blocking buffer was added to singular wells in duplicate (for the end point titration plasma was diluted up from 1/12.5 to 1/25,600), and the plates were incubated at room temperature for 60 min. The wells were washed again as described above, and 50µl of the biotinylated Mab HL1546 diluted at a final concentration of 1µg/ml in PBS/A, was added to the appropriate wells. The wells were incubated at room temperature for 60 min and washed as described above. Fifty microliters of alkaline phosphatase labelled streptavidin (AP Streptavidin; 1:5,000 dilution in PBS/A; Zymed Laboratories, Inc., San Francisco, Calif.) was added to each well, and the plates were incubated at room temperature for 90 min. After washing the wells four times with PBS-T, 100µl of p-nitrophenyl phosphate disodium (1mg/ml prepared in 0.01M sodium bicarbonate buffer [pH 9.6] containing 2mM $MgCl_2$; Sigma, St. Louis, Mo.) was added to each well and the plates were incubated in the dark at room temperature for 90 min. The OD_{405} of each well was measured

in an ELISA plate reader (EAR 400 AT; SLT-Labinstruments, 5082 Groedig/Salzburg, Austria).

Initially, a SN negative plasma sample from a Mediterranean tortoise from France, served as a negative control. Pooled plasma from five Mediterranean tortoises SN positive for herpesvirus antibodies, and having clinical signs consistent with herpesvirus, served as the positive. Subsequently, plasma collected at time zero from the herpesvirus SN and culture negative clinically healthy spur-thighed tortoises that were in the immunization study (see above), served as the "gold" negative control for determining the cutoff for positive samples. Positive and negative controls were included on each plate to determine interplate variation. Antibody level was expressed as the OD_{405} of the sample. Plasma was considered positive for tortoise herpesvirus specific antibody, when the OD_{405} of the samples was greater then the mean of the OD_{405} of the negative background plus three standard deviations.

Serum Neutralization

The plasma samples collected from the Mediterranean tortoises in the immunization study were tested for the presence of serum neutralizing antibodies directed against both the HV4295/7R/95 and HV1976 herpesvirus isolates, following an established protocol (Beard, 1989). Briefly, in a 96 well flat bottom plastic sterile plate, 25 µl of DMEM/F12 enriched with 5% FBS and antibiotic and

antimycotic were added to each well, 25 µl of each plasma sample (previously inactivated complement at 56°C for 30 mins) was added to each well and diluted 1/2 to 1/1024 using a multichannel pipette (Brinkmann, Germany). Twenty five microliters of either viral suspensions from one of the two herpesvirus isolates (HV4295/7R/95 and HV1976) containing 100 TCID50, were added to each well of the plate (a separate plate was used for each herpesvirus isolate tested) and incubated at 28.5 °C for two hours, followed by the addition of 50µl of freshly sub-cultured TH-1 cells at a concentration of $1.6x10^5$/ml. The viral suspensions used for the serum neutralization were titered on a separate plate. The plates were read after 11-14 days and the serum neutralizing titer was determined as the last dilution that did not show any cytopathic effect. Additionally the 175 sera samples from France were tested in another laboratory in Europe, using a similar technique using three different herpesviral isolates (HV2245, HV770, HV17/96).

A random sample of five SN positives (K 101, K102, K103, K104, K105) and five SN negatives (K1, K2, K3, K4, K5) of the samples tested in Europe were also tested for SN using the HV4295/7R/95 and HV1976 herpesvirus isolates utilizing the procedure described above.

Rabbit Hyperimmune Serum

Rabbit hyperimmune sera were raised against herpesvirus isolates HV4295/7R/95 and HV1976. Two specific pathogen free (SPF) rabbits were

immunized against HV4295/7R/95 and two other SPF rabbits were immunized against HV1976. An initial dose of 100μg purified viral antigen was administered to each rabbit. An equal amount of purified viral antigen was used to booster the rabbits in the study for a total of eight times (every other week for a total of 16 weeks). The immunization procedure was performed by Pel-Freez Biologicals (Rogers, AK).

Immunoblotting

Hyperimmune tortoise and rabbit sera raised against either HV4295/7R/95 or HV1976 herpesvirus isolates were tested for their ability to detect viral proteins. Infected and uninfected TH-1 cells were used as antigens. All the samples were diluted to a final concentration of 80μg total protein. An equal volume of 2x sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) sample buffer containing bromophenol blue and 5% mercaptoethanol was added to each sample (Hames and Rickwood, 1981). Samples were heated at 95 °C for 5 minutes immediately before loading them on the gel.

Samples were separated by SDS-PAGE under reducing conditions by using a pre-cast 10% Tris-glycine "curtain" gel (Bio-Rad, Hercules, CA). The proteins were then electrophoretically transferred from the gel to a Trans-Blot Transfer Medium nitrocellulose membrane (0.2μm) (Bio-rad, Hercules, CA) (Harlow and Lane, 1988; Towbin et al., 1979) using the Bio-rad miniprotean II apparatus (Bio-

rad, Hercules, Ca) according the manufacturer's instructions. A Tris-Glycine buffer (pH 8.3) (Bio-rad, Hercules, CA) in 20% methanol was used as transfer buffer. The blotting time was 45 min at a constant voltage of 75V. As soon as the transfer was complete, the nitrocellulose was blocked with PBS containing 5% nonfat dry milk and the mixture was incubated at 4°C overnight. The nitrocellulose was then washed three times (5 min per wash) with PBS-T and placed into a Fisher nylon cutter (Fisher, Pittsburgh, PA) to obtain 25 separate strips. A total of 300µl hyperimmune plasma diluted in blocking buffer (diluted 1/25 tortoise; 1/500 rabbit) was loaded in each channel of the incubation tray and then it was incubated at 28 °C (tortoise sera) or 37 °C (rabbit sera) on a Hematek shaker (Miles laboratories Elkhart, IN) overnight. The strips were then washed three times for ten minutes with PBS/T followed by the addition of 300µl of biotinylated mouse anti-Mediterranean tortoise immunoglobulin Mab HL1546 (diluted 1µg/ml in PBS/A) into each channel and incubated for 120 min at room temperature on a shaker. After three more washes with PBS/A, the nitrocellulose strips were incubated either with AP strepavidin (diluted 1/5000; Zymed Laboratories, Inc., San Francisco, CA; in PBS/A) (tortoise) or with AP steptavidin labeled goat anti-rabbit antibody (1/10,000; Sigma, St Louis, MO) (rabbit) at room temperature for 60 min. After three more washes with PBS/T, the blot was developed with substrate buffer [0.1 M Tris HCl, 1mM MgCl2 (pH8.8)] containing 44µl of nitroblue tetrazolium chloride (NBT) and 33 µl of 5-bromo-chloro-3-indolyphosphate p-toluidine salt (BCIP) per 10 ml of substrate buffer. NBT and BCIP were obtained commercially (Promega, Madison, WI).

Statistical Analysis

The McNemar test was used for comparing the ELISA test to the SN test. A P value of 0.05 was considered significant.

Results

Determination of ELISA parameters

End Point Titrations. Figures 1a and 1b show the end point titration curves obtained for anti-herpesvirus antibodies titers. The antibody titer of the pre-immunization and SN negative tortoises was never higher than OD_{405} 0.4 at the lowest dilution (1/12.5). The ranges of the OD_{405} reading for the negative plasma samples were 0.155 and 0.086 at 1/12.5 and 1/25 final dilution respectively when tested against the HV4295/7R/95. The ranges of the OD_{405} reading for the negative plasma samples on HV1976 were 0.099 and 0.067 at 1/12.5 and 1/25 dilutions. When a plasma dilution of 1/25 was used, the positive plasma samples showed an average reduction of the OD_{405} reading of 0.09 and 0.08 when tested respectively against HV4295/7R/95 and HV1976. A plasma dilution of 1/25 was selected for the test.

The mean OD_{405} reading value (0.2014) of the plasma samples collected from the spur-thighed tortoises in the immunization study at time zero was used in

determining the cutoff for the positive samples in the ELISA assay. The cut-off was established as the mean OD_{405} reading value (0.2014) plus three times the standard deviation (0.094). This computation resulted in a final cut-off value of 0.48 (OD_{405}).

Evaluation of Plasma Samples by Elisa and Serum-neutralization

A total of 175 plasma samples collected from Mediterranean tortoises in a rehabilitation facility in France previously tested for the presence of serum-neutralizing antibodies were tested with the ELISA assay. Twenty percent of the samples tested positive with the SN, 80% tested negative (Table 1a and 1b). When the same samples were tested by ELISA, 24.6% tested positive either for the European (HV4295/7R/95) or the American (HV1976) herpesvirus isolates, while 21.1% tested positive for both the European and the American herpesvirus isolates (Table 1b). When tested against both the American and European isolates, 75.4% were negative (Table 1b). Four samples (2.3%) tested positive only for the American isolate (HV1976) while two samples (1.1%) tested positive only for the European isolate (HV 4295/7R/95) (Table 1b). Three samples (1.7%) that tested positive for both of the antigens (HV4295/7R/95 and HV1976) with the ELISA assay were negative for the presence of SN antibodies (Table 1a). Four samples (2.3%) that tested negative for the presence of serum-neutralizing antibodies were positive using the ELISA assay for the American isolate (HV1976) only (Table 1b). One sample (0.6%) that was negative by SN,

tested positive by ELISA against the European isolate (HV4295/7R/95), and negative when using the American isolate (HV1976). One sample (0.6%) positive for the presence of SN antibodies, was positive by ELISA when tested with the European isolate (HV4295/7R/95) but resulted negative when tested with the American isolate (HV 1976) (Tables 1a and 1b). Arbitrarily, a sample was considered positive with the ELISA assay when seropositive when using both viral antigens in the test.

Table 1a

	ELISA positive	ELISA negative	Totals
SN positive	34	1	35
SN negative	3	137	140
Totals	37	138	175

Table 1b

ELISA test against HV4295/7R/95 and HV1976					Serum-neutralization test against HV770, HV2245, HV17/96*	
Positive for both of the antigens (HV4295/7R/95 and HV1976)	Positive for the HV4295/7R/95 antigen only	Positive for the HV1976 antigen only	Positive either for one of the antigens	Negative for both of the antigens (HV4295/7R/95 and HV1976)	Positive for all of the three antigens (HV770, HV2245,HV17/96)	Negative for all of the three antigens (HV770, HV2245,HV17/96)
37	2	4	43	132	35	140

*The SN titer against this isolate (HV17/96) was not available for sample K20

The OD_{405} reading of the samples positive both by ELISA and SN had a mean value of 1.57 and 1.53 when tested respectively against the HV4295/7R/95 and HV1976 viral isolates (34 samples). The negative OD_{405} reading assumed a mean value of 0.172 and 0.168 (132 samples) when tested respectively against the HV4295/7R/95 and HV1976 viral isolates. In the calculation of these values we did not consider the samples that resulted positive by ELISA and negative by SN (3) or those samples positive by ELISA only for one of the viral antigens used (6) in the assay (see Tables 1a and 1b above).

For determining the epidemiological parameters of specificity, sensitivity, positive and negative predictive value and prevalence (Baldock, 1988), the SN test was considered the gold standard. The values are summarized in Table 2.

Table 2. Epidemiological parameters determined for the ELISA test (computed according Baldock, 1988)

Parameter	Value
Specificity	98%
Sensitivity	97%
Positive Predictive Value (PPV)	92%
Negative Predictive Value (NPV)	99%
Seroprevalence	21%
Prevalence	20%

Immunoblot Evaluation of Viral Antigens

Infected and not infected TH-1 cells were evaluated for their immunoblot profiles. As shown in Figure 2, hyperimmune tortoise sera raised either against HV1976 or HV4295 detected a limited number of proteins spanning from about 20 KDa to more than 250 KDa . Two bands detectable at 140 and 75 KDa in the uninfected Th-1 cells and weakly in the viral infected cells were considered non specific, as they were recognized by the secondary biotinylated antibody HL1546 in the

absence of the primary antibody and by the pre-exposure tortoise sera (data not shown). A band at approximately 200 KDa was recognized by both antisera against both viruses. A band corresponding to a molecular weight higher than 250 KDa was detectable by both antisera, but only in the HV4295/7R/95 infected cells. A very weak band at about 20 KDa was detectable both in the HV1976 and the HV4295/7R/95 infected cells when the anti HV1976 tortoise polyclonal sera was used. A similar but even weaker antibody reaction, was detectable in the HV4295/7R/95 infected cells too when the anti HV 4295/7R/95 tortoise polyclonal sera was used.

Rabbit polyclonal (Fig. 3) anti-HV1976 and anti-HV4295/7R/95 sera showed an antibody reaction against a group of bands spanning from about 20 to 35 Kda for both viral isolates. Anti-HV1976 showed a stronger antibody reaction against these bands than anti-HV4295/7R/95. A low molecular weight band (approximately 16 KDa) was detected with anti-4295/7R/95, but it was not detected when using anti-HV1976 as the primary antibody. Both sera showed a strong antibody reaction against all proteins from both isolates spanning from about 50 KDa to more than 250 KDa, with two major bands recognizable at approximately 75 and 140 KDa (the former is weakly recognized in the uninfected TH-1 cells).

Both the tortoise hyperimmune sera and rabbit anti-herpes polyclonal showed an antibody reaction against a protein of approximately 20 KDa of molecular weight.

Immunization Study

Clinical Findings. All four Mediterranean tortoises immunized with HV4295 or HV 1976, either IM or IN, showed clinical signs consistent with herpesvirus infection. Mild to severe muco-purulent bilateral conjunctivitis with swelling of the eyelids was observed during the study. The clinical signs seemed to be more severe when the tortoises were immunized IM, while the tortoises immunized intranasally showed milder conjunctivitis with limited ocular discharge. Both of the tortoises immunized IM showed more severe and prolonged recurrent conjunctivitis during the study. No other major clinical signs than conjunctivitis were detectable. All the tortoises gained weight during the study. All but one of the tortoises with clinical signs, remained seronegative for anti-herpesvirus antibodies both with the ELISA and the SN test.

Serology. The plasma from tortoises immunized with HV1976 (#2 and #4 respectively) had an OD_{405} reading higher than 0.48 at four weeks after immunization when tested using HV4295/7R/95. When these samples were tested against HV1976 only, T#4 resulted positive (T#2 OD_{405} reading against HV1976 [OD_{405} =0.46]) (Fig.4a and 4b). At that same point in time neither of the two tortoises immunized with HV4295/7R/95 showed an OD_{405} reading higher than 0.48. Seroconversion was detected in tortoise #3 at seven weeks post

immunization. At this time, tortoises #2, 3 and 4 showed an OD_{405} reading higher than 0.48 when using both viral antigens (HV4295/7R/95 or HV1976). Tortoise #1 never seroconverted, either by ELISA or the SN. Beginning at 9 weeks post exposure, the OD_{405} reading for plasma samples from tortoises #2, 3 and 4 was never lower than 1.3 when tested against either viral antigens (HV4295/7R/95 or HV1976).

Table 3. Mean OD_{405} values calculated for the immunized tortoises after 6/7 weeks from exposure (respectively for the intranasal and IM) when tested against HV4295/7R/95 and HV1976 viral antigens.

Tortoises	OD_{405} (HV4296/7R/95)	OD_{405} (HV1976)
#1 HV4295/7R/95 infected	0.168	0.154
#2 HV1976 infected	1.72	1.58
#3 HV4295/7R/95 infected	1.59	1.51
#4 HV1976 infected	1.63	1.51
#5 (control)	0.16	0.25

A high degree of cross-reactivity was detected in all the hyperimmune sera for both of the herpesvirus isolates used as antigens (HV1976 and HV4295/7R/95). Tortoise #1, despite an additional immunization (IN) performed at three months

after the first immunization with HV4295/7R/95, never seroconverted when measured by ELISA or SN.

SN results paralleled the ELISA findings and are summarized in Tables 4a-4b (SN) and Figures 4a-4b (ELISA). For all the tortoises in the immunization study except for tortoise #1, serum-neutralizing antibodies directed against both herpesviral isolates were detected. SN titers directed against both HV1976 and HV4295/7R/95 were detected after 9 weeks post immunization for Tortoise #2 while no serum neutralizing activity was ever detected in plasma of Tortoise # 1. SN titers for plasma of Tortoise #2 plasma directed against the homologous isolate, ranged between 1/8 and 1/16, while for the heterologous isolate it ranged between 1/8 and 1/64. The highest titer detected for tortoise # 2 (1/64), was recorded for the first time 23 weeks post immunization.

Both of the tortoises immunized IM were positive for SN antibodies. While tortoise # 4 sera was SN positive both against the homologous (HV1976) and the heterologous (HV4295/7R/95) isolate, tortoise # 3 was SN positive only for the homologous isolate (HV4295/7R/95) up to 21 weeks post immunization. After 25 weeks post immunization the first SN titer for the heterologous isolate (HV1976) was detected. For tortoise # 3, SN titers were never higher than 1/8, detected against the heterologous isolate at 34 weeks post immunization. The highest titer for tortoise # 3 against the homologous isolate (1/4) (HV4295/7R/95) was detected for the first time, after 9 weeks post infection.

The SN titer for tortoise # 4 ranged from 1/4 to 1/64 against both the heterologous (HV4295/7R/95) and the homologous (HV1976) isolate. The highest SN titer for tortoise # 4 (1/64) and was detected against the heterologous isolate (HV4295/7R/95) after 21 weeks post immunization and against the homologous isolate (HV1976) after 34 weeks post immunization.

The SN results obtained from the European tortoise collection reflected the same immunological cross-reactivity pattern that was seen in the tortoises in the immunization study. All SN positive samples collected in Europe reacted against all three isolates used in the test (HV770, HV2245, HV17/96). The most interesting difference between the SN titers detected from the tortoise samples from France and those in the immunization study was the higher titer seen in the samples from France. One sample from France had a titer of 1/726 and several had a titer of 1/362.

ELISA and Serum Neutralization Cross reactivity

By ELISA and SN, immunological cross reactivity was recorded between the two herpesviral isolates. In order to better evaluate this cross reactivity, a random sample of 10 sera (5 SN negative and 5 SN positive for HV770, HV2245, HV17/96) was selected from the French sample group and tested for SN against the herpesviral isolates used as antigens in the ELISA test (HV4295/7R/95 and HV1976). SN results for these samples (using HV1976 and HV4295/7R/95)

correlated 100% with the SN results using the European herpesvirus isolates (HV770, HV2245, HV17/96). SN titers ranged from 1/8 to 1/128 and from 1/16 to higher than 1/256 when tested respectively against HV4295/7R/95 and HV1976. These results demonstrated a high degree of cross reactivity between these five herpesvirus isolates (HV770, HV2245, HV17/96, HV1976, HV4295/7R/95). These results were consistent with the cross reactivity detected between the herpesviral isolates HV4295 and HV1976, both by ELISA and SN.

Reproducibility of the ELISA

An estimate of the reproducibility of the ELISA is given in Table 5. Interassay reproducibility was determined by calculating the mean OD_{405}, the standard deviation, and the range for the negative and positive control of 5 ELISAs over a time period of one month using the same batch of HV4295/7R/95 and HV1976 antigens, plasma, and Mab HL1546 (Table # 5).

Table #5

Plasma samples	Antigens	Dilutions	Mean OD_{405}	SD±	Range
Neg. Control	HV4295/7 R/95	1/25	0.173	0.0063	0.159
Pos. Control	HV4295/7 R/95	1/25	1.596	0.0019	0.043
Neg. Control	HV1976	1/25	0.157	0.01	0.024
Pos. Control	HV 1976	1/25	1.581	0.12	0.341

*Plasma samples used as : negative control: K55

positive controls: MB1, MB34, MB42, MB40, TGT

Discussion

Herpesvirus is being recognized an increasingly important pathogen in chelonians. In recent years several reports have called attention to the frequent association of a disease characterized by stomatitis-rhintis with the presence of herpesvirus-like particles. Both the conservation importance of the tortoises genus *Testudo*, and their extreme popularity as pets in Europe and in the US, were important factors for the development of the first ELISA based test for detecting herpesvirus exposure in tortoises.

Serum Neutralization (SN) has been considered the "Gold Standard" for the detection and quantification of antiviral antibodies (Murphy et al, 1999) and has been used for detecting herpesvirus exposure in tortoises (Frost and Schmidt, 1997). The limitations of SN as a sero-epidemiological tool include its laborious procedure, the long time required to determine the final result (10-14 days) and the absence of a universally recognized reference isolate. In contrast the ELISA test offers the advantage of a one-day procedure with an overall simplicity of execution.

In order to evaluate the specificity and the sensitivity of the ELISA test, we tested 175 plasma samples obtained from spur-thighed and Hermann's tortoises in a rehabilitation facility in France where herpesvirus was endemic. These samples had been previously evaluated by a colleague in Germany for the presence of anti-herpesvirus SN antibodies using three herpesvirus European isolates isolated from symptomatic tortoises (HV770, HV2245, HV17/96) in Europe (Blahak pers. com.). The results (Table 1a and 1b) suggest a good correlation between the ELISA and the SN test. All the samples that tested positive for SN were positive also for ELISA. The only exception was one SN positive sample which was positive only in the ELISA test for one of the isolates (HV4295/7R/95) (Table 1a). For a limited number of samples (six equal to 3.4% of the population studied) we observed a positive OD_{405} reading either for one or the other of the viral antigens used in the test (HV4295/7R/95 and HV1976), but not for the both of them. This result might be related to antigenic characteristics of the two

different isolates. The great majority of the samples showed cross-reactivity, suggesting the presence of conserved antigenic motifs among the five herpesviral isolates used either in the ELISA or in the SN test. In determining sensitivity and specificity values of the ELISA, we used a conservative approach and considered all the samples as positive when tested positive using both antigens. When this was done there was a total of 138 negative and 37 positive samples detected by ELISA, compared to 140 negative and 35 positive samples detected by SN. When we used SN as a test for the "true epidemiological status assessment" of herpesvirus exposure in tortoises, then the ELISA had a specificity of 97% and a sensitivity of 98%. The seroprevalence determined by ELISA was 20% when adjusted for sensitivity and specificity.

Two additional important serologic parameters were the positive and the negative predictive values (PPV; NPV). The PPD is the probability that the subject has the disease when the test result is positive, while the NPV is the probability that the subject is disease free when the test result is negative. The value of PPV and of NPV determined for the ELISA test were respectively 92% and 98%. In our study we considered the SN test as the "Gold Standard" and the results obtained from this test were considered "true values". Traditionally when a new diagnostic test is compared to a gold standard, the two tests need to be biologically independent, that is to say that they measure different targets (Baldock, 1988). In this study while the SN test measured the presence of herpesvirus SN antibodies, the ELISA test theoretically measured all the possible

sub-classes of IgY directed against herpesvirus. Statistical analysis showed no significant difference between the results of the ELISA and SN tests ($P>0.05$)

When looking at the "crude" OD_{405} readings, we observed that the mean values obtained in the immunization study and from the plasma samples of the tortoises from France, were comparable. While the mean positive value calculated for the tortoise collection from France ranged from 1.53 to 1.57, the same values ranged from 1.63 to 1.72 and from 1.51 to 1.58 (Table 3) for the tortoises in the immunization study when tested respectively using HV4295/7R/95 and HV1976. The mean negative values ranged from 0.168 to 0.172 in the tortoise collection from France. In the immunization study these values ranged from 0.16 to 0.168 and from 0.154 to 0.25 when tested respectively against HV4295/7R/95 and HV1976 (Table 3). These results might depend again on antigenic similarities between the different herpesviral isolates or on a specific immunologic response elicited by the herpesvirus in tortoises.

An interesting difference was observed between the SN positive samples from France and those from our immunization study. None of the SN positive samples from our immunization study was higher than 1/64, while much higher values were detected in the SN positive samples from Europe, where values of 1/362 and higher were not uncommon. There may be several explanations for this difference. In our immunization study, tortoises were exposed to the virus one single time (except for tortoise #1 which was injected a second time intransally

two months after the first exposure with 15,000 TCID50 of HV4295/7R/95) while we have no data about re-infection that could have happened over time in the tortoise collection in the rehabilitation facility in France. Second, we immunized the tortoises in our study with a viral dose of 15,000 TCID50, while in the natural transmission of the virus in the rehab facility in France, we do not know if the tortoises were exposed to a higher viral load. Possibly the immunological response is dose dependent. Furthermore, we used two isolates that were passed several times in culture, while the isolates that infected the tortoises from Europe were by definition "wild types". It is possible that the "laboratory" isolates we used were attenuated, and less immunogenic compared to the virus that infected the tortoises in France. This possible attenuation may account for the observed minimal stimulation of SN antibody production. Interestingly, when 10 random samples from France were tested against the two herpesviral isolates used as antigens in the ELISA test (HV4295/7R/95 and HV1976), the SN titers obtained were higher than the titers from the immunized animals with the homologous isolates. These data support this hypothesis.

In order to validate the ELISA, we immunized a group of five spur-thighed tortoises using the same herpesvirus isolates that were used as the antigen in the test (HV4295/7R/95 and HV1976). There are anecdotal reports of a higher resistance to disease caused by herpesvirus in spur-thighed tortoises compared to Hermann's tortoises and because of this we decided to use the former in the immunization study. When we evaluated the plasma samples from all the

Hermann's tortoises from France, [59 out of 175 (33.7%)] none of them resulted seropositive for anti-herpesvirus antibodies by SN and ELISA. This is consistent with the report by Frost and Schmidt (1997) in which Hermann's tortoises and Russian tortoises (*T. horsfieldii)* were found to be "low serologic reactors" with higher mortality when compared to spur-thighed tortoises and marginated tortoises (*T.marginata)*, that generally showed a higher seroprevalence and a low mortality during herpesvirus epizootics. This data could suggest a slow or delayed antibody response or an unresponsive immune system in Hermann's tortoises.

The amount of live virus used for immunizing the tortoises in the study, was deliberately not very high (15,000 TCID50), because we wanted the tortoises to survive the immunization and develop an immune response. Both a natural route (intranasal) and an experimental route (IM) were used.

The results of this study indicated that seroconversion was not detectable earlier than 4 weeks post exposure when using either the ELISA or SN test. This response was earlier than that observed in desert tortoises (*Gopherus agassizii*; 8 weeks) when immunized with mycoplasma (Schumacher et al., 1993). Additionally, the ELISA test detected the presence of anti-herpesvirus antibodies 2 to 5 weeks earlier than the SN test. These results are not surprising since ELISA can detect antibodies binding to multiple antigenic determinants of a virus, while the SN test detects anti-herpesvirus antibodies which are biologically active in neutralization and directed against surface glycoproteins. The ELISA was also

found to have high specificity, sensitivity and both high positive and negative predictive values. There also was good consistency in the test results.

Immunological cross reactivity was observed when comparing the two herpesvirus isolates used in our study with three additional isolates used in Germany. This has been reported for other herpesviruses such as Varicella Zoster herpesvirus (VZV), which is well known for showing a large degree of cross reactivity in serological tests (Arvin, 1996). Frost and Schimdt (1997) concluded that given the serological relatedness of the tortoise herpesviruses isolates, it would be sufficient to employ one single isolate for the purpose of SN testing. However, the existence of possible serogroups of tortoise herpesvirus has been discussed (Biermann, 1995). The findings in our study may be indicative of major antigenic similarities shared between the two herpesviruses isolates used. This is supported by a good correlation of SN results for the samples from France when tested using either three herpesviral isolates from Germany or with the two isolates used in our ELISA.

The immunization study also demonstrated that anti-herpesviral antibodies were detectable in tortoises for a minimum of nine months after a single exposure when using either ELISA or SN. The ELISA results did not indicate any variation in the antibody level after six and seven weeks post-exposure (respectively for the IN and IM). However more variation was seen using the SN test.

Some of the clinical signs reported in the literature for tortoises with herpesvirus infection were seen in our immunized tortoises. Recurrent serous to muco-purulent bilateral or unilateral conjunctivitis was observed in tortoises following a single immunization. Another herpesvirus, bovine herpesvirus 1, responsible for infectious bovine rhino-tracheitis (IBR), may induce the disease in a bovine herd where the only detectable clinical sign is a unilateral or bilateral conjunctivitis (Murphy et al., 1999/b). A more severe conjunctivitis was observed in the two tortoises immunized IM compared to those immunized IN. An IN route may allow the host to better react to the pathogen.

Preliminary immunoblotting results showed several bands recognized by the hyperimmune tortoise and rabbit sera on herpesviral infected TH-1 cells and not recognized in uninfected TH-1 cells. One of the non-specific band corresponded to a protein of an approximately molecular weight of 75 Kda detected both with hyperimmune tortoise and rabbit sera is likely a response to fetal bovine albumin (Fig. 2 and 3). The high molecular weight bands may correspond to surface viral glycoproteins, at different level of glycosylation.

Noteworthy was the detection by both the rabbit and tortoise hyperimmune sera of an approximately 20 KDa polypeptide. This band was strongly recognized by the hyperimmune rabbit sera while it was very weakly detectable with the hyperimmune tortoise sera. Since the rabbits were immunized and boostered repeatedly with a total amount of 900 μg of purified viral protein and the tortoises received one single dose of the virus (15,000 TCID50 total), this is not surprising.

In summary, this study reports the development of an ELISA for detection of herpesvirus exposure in Mediterranean tortoises. Without affecting the consistency of the results, the ELISA was far more practical compared to the more laborious and time consuming SN. The transmission study demonstrated that the ELISA detects seroconversion earlier than SN. This is extremely important for seroepidemiological studies. The major cumbersome component of the ELISA is the need to continually harvest virus used as the antigen in the test. The test could be further refined by the replacement of whole virus with a recombinant viral protein. This would allow a better standardization of the test, and a concomitant reduction of the cost of antigen production. Even with some of the problems associated with antigen preparation, the ELISA has broad application in seroepidemiological investigations of the prevalence of herpesvirus exposure in wild and private collections of tortoises.

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References

1. Andreas, E.M., and H. Ambrosius. 1989. Surface immunoglobulin on lymphocytes of the tortoises Agrionemys horsfieldii. Dev. Comp. Immunol. 13: 167-175.

2. Arvin, A.M. 1996. Chapter 79: Varicella-Zoster Virus, p. 2547-2585. In B. N. Fields, D. M. Knipe, P. M. Howley (ed.), Virology, 3rd ed., Lippincot-Raven publisher, Philadelphia, PA.

3. Baldock, F. C. 1988. Epidemiological Evaluations of Immunological tests, p 90-95. In G. W. Burgess (ed.), ELISA Technology in Diagnosis and research, Graduate School of Tropical Veterinary Science James Cook University of North Queensland, Townsville, Australia.

4. Beard, C. W.1989. Serological Procedures, p 195-196. In H. G. Purchase, L. H. Arp, C.H. Domermuth and J. E. Pearson (ed.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens 3rd ed. American Association of Avian pathologists. Kendall/Hunt Publishing Co., Dubuque, IA.

5. Biermann, R. 1995. Isolierung und Charakterisierung von Herpesviren bei Landschildkroten. Veterinary Medical Dissertation, Justus Liebig University, Giessen, Germany.

6. Cox, W. R., W.A. Rapley, and I. K. Barker. 1980. Herpesvirus-like Infection in a painted turtle (*Chrysemys picta)*. J Wild Dis 16:445 -449

7. Drury, S. E. N., R.E. Gough, S. McArthur, et al. 1998. Detection of herpesvirus-like and papillomavirus associated with diseases of tortoises. Vet Rec 143:639

8. Drury, S. E. N., R.E. Gough, S. McArthur. 1999. Isolation and identification of herpesvirus and papillomavirus from tortoises in Great Britain, p. 69-75. In Proceedings of the 6th ARAV Conference, Columbus, Ohio.

9. Frye, F. L., S. L. Oshiro, F. R. Dutra, J. D. Carney. 1977. Herpesvirus like Infection in two pacific pond turtles. J Am Vet Med Assoc 171:882-883

10. Frost J. W., A. Schmidt. 1997. Serological evidence of susceptibility of various species of tortoises to infection by herpesviruses. Verh Ber Erkrg Zootiere, 38: 25-27.

11. Goding J. W. 1986. Monoclonal antibodies, principles and practice, 2nd ed., p 263-265. Academic Press, Inc., Orlando, Fl.

12. Hadge, D, H. Fiebig. and, H. Ambrosius. 1980. Evolution of low molecular weight immunoglobulins. I .Relationship of 7S immunoglobulins of various vertebrates to chicken IgY. Dev Comp. Immunol. 4:501-514.

13. Hames, BD, and D. Rickwood (ed.). 1981. Gel electrophoresis of proteins. Oxford University Press, New York.

14. Harbeck, R. J., and P.C. Giclas. 1991. General ELISA procedure for detection of miscellanous antigens and antibodies, p. 173-183. In R.J. Harbeck and P.C. Giclas, Diagnostic immunology laboratory manual. Raven Press, New York.

15. Harlow E., and D. Lane. 1988. Immunoblotting, p.473-510. In Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

16. Harper P. A. W., D. C. Hammond, and W. P. Heuschele, 1982. A Herpesvirus-like agent associated with a pharyngeal abscess in a desert tortoise. J Wild Dis.18: 491-494

17. Heldstab, A, and G. Bestetti. 1989. Herpesviridae causing glossitis and meningoencephalitis in land tortoises (*Testudo hermanni*). Herpetopathologia. 1: 5-9

18. Hierholzer, J. C., and R. A. Killington. 1996. Virus Isolation and Quantification, p. 25-46. In . W. J. B. May, and H. O. Kangro (ed.), Virology Methods Manual. Academic Press, San Diego, Ca.

19. Jacobson, E. R., J. M. Gaskin, H. Wahlquist. 1982. Herpesvirus-like infection in map turtles. J Am Vet Med Assoc 181: 1322-1394

20. Jacobson, E. R., S. Clubb, J. G. Gaskin, et al. 1985. Herpesvirus-like infection in Argentine tortoises. J Am Vet Med Assoc 187:1227-1229

21. Jacobson, E.R, J. M. Gaskin, M. Roelke, et al. 1986. Conjunctivitis, tracheitis, and pneumonia associated with herpesvirus infection in green sea turtles. J Am Vet Med Assoc 189:1220-1223

22. Jacobson, E. R., C. Buergelt, B. Williams. 1991. Herpesvirus in cutaneous fibropapillomas of the green turtle (*Chelonia mydas*). Dis Aquat Org 12: 1-6

23. Kabish D, J. W. Frost. 1994. Isolation of herpesvirus from *Testudo hermanni* and *Agrionemys horsfieldii*. Verh Ber Erkrg Zootiere 36: 241- 245

24. Kao K. J., and P.A. Klein. 1986. A monoclonal antibody-based enzyme-linked immunosorbent assay for quantitation of plasmathrombospondin. J Clin pathol 86: 317-323.

25. Leslie, G. A., and L. W. Clem. 1969. Phylogeny of immunoglobulin structure and function. III. Immunoglobulin of the chicken. J Exp Med 130: 1337-1352.

26. Liddell, J.E. , and A. Cryer. 1991. A practical guide to monoclonal antibodies. John Wiley & Sons, Ltd., Chichester, England.

27. Marschang, R. E., M. Gravendyck, and E. F. Kaleta. 1997. Investigation into virus isolation and the treatment of viral stomatitis in *T. hermanni* and *T. graeca*. J Vet Med. Series B. 44: 385- 394

28. Marschang, R. E., H. Posthaus , M. Gravendyck, et al. 1998. Isolation of Viruses from Land Tortoises in Switzerland, p. 281-284. *In* Proceedings of the AAZV and AAWV Joint conference. Omaha, Nebraska.

29. Marschang, R. E. 1999. Evidence for a new herpesvirus serotype associated with stomatitis in Afghan Tortoises, *Testudo horsfieldii*, p. 77-79. In Proceedings of the 6th ARAV Conference, Columbus Ohio.

30. Murphy, F. A., E. P. J. Gibbs, M. C. Horzinek, M. J. Studdert. 1999. Chapter 12: Laboratory Diagnosis of Viral Diseases, p. 193-224. In Veterinary Virology. 3rd ed. Academic Press.

31. Murphy, F. A., E. P. J. Gibbs, M. C. Horzinek, M. J. Studdert. 1999. Chapter 18: Herpesviridae, p. 301-326. In Veterinary Virology. 3rd ed. Academic Press.

32. Muro, J., A. Ramis , J. Pastor , L. Velarde , J. Tarres , S. Lavin. 1998. Chronic rhinitis associated with Herpesviral Infection in captive spur-thighed tortoise from Spain. J Wild Dis. 34:487-495

33. Rebell, H., A. Rywlin, and H. Haines. 1975. A herpesvirus-type agent associated with skin lesions of green turtles in aquaculture. Am J Vet Res 36:1221-1224

34. Schumacher I.M., M. B. Brown, E. R. Jacobson, B. R. Collins, P. A. Klein. 1993. Detection of Antibodies to a Pathogenic Mycoplasma in Desert Tortoises (Gopherus agassizii) with Upper Respiratory Tract Disease. J of Clin Micro, 31: 1454-1460

35. Simrell, C. R. and P. A. Klein. 1979. Antibody response of tumor-bearing mice to their own tumors captured and perpetuated as hybridomas. J. Immunol. 123: 2386-2394.

36. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets; procedure and some applications. Proc. Natl. Aad. Sci. USA 76:4350-4354.

37. Une Y, K. Uemura, Y. Nakano, J. Kamiie, T. Ishiabashi, and Y. Nomura. 1999. Herpesvirus infection in Tortoises (*Malacochersus tornieri* and *Testudo horsfieldi*). Vet Path 36: 624-627.

38.Warr, G. W., K. E. Magor and D. A. Higgins. 1995. IgY: Clues to the origins of modern antibodies. Immunology Today, 16: 392-398.

Figure legends

Figure 1 End point titration values when using HV4295/7R/95 (1a) and HV1976 (1b) as the antigens.

Anti-herpesvirus antibody titers were measured for pre-immunized healthy Mediterranean tortoises, 9 weeks post immunized tortoises (from the immunization study), a pool of SN negative tortoise plasma (not included in the immunization study [K8, K9, K12, K14]) and a pool of SN positive tortoise plasma (not included in the immunization study [K16, K82, K83, K104]). The values reported on the Y axes are expressed as OD405 readings, while on the X axis, reciprocal of plasma dilution values are indicated.

Figure 2 Comparision of the antigens of 4295/7R/95 and HV1976 using hyperimmune tortoise sera (antibodies reactions in lanes 1, 2 and 3 are obtained using tortoise sera raised against HV4295/7R/95; antibodies reactions in lanes 4, 5 and 6 are obtained using tortoise sera raised against HV 1976) (lane 1 = HV1976; lane 2 = HV4295/7R/95; lane 3 = TH-1 cell lysate; lane 4 = HV1976; lane 5 = HV4295/7R/95; lane 6 = TH-1 cell lysate)

Figure 2 shows the western blot results for the hyperimmune tortoise sera. Few antibody reactions are detectable, and a very faint band of approximately 20 KDa is observable in the infected cells when stained using anti-HV1976 polyclonal tortoise sera. An antibody reaction with a similar molecular weight is detectable also in the HV4295/7R/95 infected cells, using anti-HV4295/7R/95 polyclonal tortoise sera. An antibody reaction of approximately the same size is detectable also in figure 3 in the infected cells. The arrows show the approximate molecular weight of the different antibody reactions

Figure 3 Comparision of the antigens of 4295/7R/95 and HV1976 using rabbit polyclonal antibodies raised against HV1976 (STF) (lane 1, 2, and 3) and HV4295/7R/95 (FOF) (lane 4, 5 and 6) (lane 1 = HV1976; lane 2 = HV4295/7R/95; lane 3 = TH-1 cell lysate; lane 4 = HV1976; lane 5 = HV4295/7R/95; lane 6 = TH-1 cell lysate)

Several low molecular weight antibody reactions can be seen in lanes 1, 2, 4 and 5 and absent in lane 3 and 4 (uninfected TH-1 cells) (2a). FOF sera was raised

against HV4295/7R/95, while STF was raised against HV1976. The arrows show the approximate molecular weight of the different bands

Figure 4. ELISA results for tortoises immunized with HV1976 and HV4295/7R/95 intranasally (4a) and IM (4b).

In the graphs depicted in figures 4a and 4b, are reported the OD_{405} readings obtained for the plasma samples collected from the tortoises in the immunization study from time 0 to 36 and 34 weeks after immunization (IN and IM inoculation respectively). Each bar corresponds to a single sampling. On the left side of the graphs are the results from the ELISA test when the tortoise plasma samples are tested against HV4295/7R/95 used as antigen, while on the right are those obtained when tested against HV1976. The values reported on the Y axis are expressed as OD405 readings. On the X axis the tortoises ID are reported along with the vaccination antigens in brackets [(HV4295/7R/95 (tortoises #1 and # 3), HV 1976 (tortoises #2 and #4), PBS (tortoise #5)]. On the top of the graphs are reported the antigens used in the ELISA test.

Table 4 SN results for tortoises immunized with HV4295/7R/95 and HV1976, IN (4a) and IM (4b).

In table 4a and 4b are reported the SN titers obtained for the plasma samples collected from the tortoises in the immunization study from time 0 to 36 and 34 weeks after immunization (IN and IM inoculation respectively). The antigens used for the immunization and for the SN test are reported on the top and the bottom

of each table respectively. A total viral amount of 100TCID50 was used in the SN test.

Table 4 a. SN data I.NI Immunization

	SN antigen HV4295/7R			SN antigen HV1976		
	Tort. C.	Tort. # 1	Tort. # 2	Tort. C.	Tort. # 1	Tort.#2
Time O	-	-	-	-	-	-
Time 2 WPI	-	-	-	-	-	-
Time 4 WPI	-	-	-	-	-	-
Time 6 WPI	-	-	-	-	-	-
Time 9 WPI	-	-	+ (1/8)	-	-	+ (1/8)
Time 11 WPU	-	-	+ (1/32)	-	-	+ (1/4)
Time 13 WPI	-	-	+ (1/32)	-	-	+ (1/4)
Time 15 WPI	-	-	+ (1/32)	-	-	+ (1/8)
Time 17 WPI	-	-	+ (1/32)	-	-	+ (1/16)
Time 23 WP	-	-	+ (1/64)	-	-	+ (1/8)
Time 27 WPI	-	-	+ (1/64)	-	-	+ (1/16)
Time 31 WPI	-	-	+ (1/8)	-	-	+ (1/16)
Time 36 WPI	-	-	+ (1/16)	-	-	+ (1/16)
Vaccination Antigen	PBS	HV4297/7 R/95	HV 1976	PBS	HV4297/7 R/95	HV 1976

Tble 4 b. SN Data I.M Immunization

	SN antigen HV4295/7R			SN antigen HV1976		
	Tort. C.	Tort. # 3	Tort. # 4	Tort. C.	Tort. # 3	Tort.#4
Time O	-	-	-	-	-	-
Time 2 WPI	-	-	-	-	-	-
Time 4 WPI	-	-	-	-	-	-
Time 7 WPI	-	-	+ (1/4)	-	-	+ (1/8)
Time 9 WPI	-	+ (1/4)	+ (1/8)	-	-	+ (1/4)
Time 11 WPU	-	+ (1/4)	+ (1/16)	-	-	+ (1/8)
Time 13 WPI	-	+ (1/2)	+ (1/16)	-	-	+ (1/16)
Time 15 WPI	-	+ (1/4)	+ (1/32)	-	-	+ (1/4)
Time 21 WPI	-	+ (1/4)	+ (1/64)	-	-	+ (1/32)
Time 25 WP	-	+ (1/2)	+ (1/16)	-	+ (1/4)	+ (1/32)
Time 29 WPI	-	-	+ (1/8)	-	+ (1/4)	+ (1/32)
Time 34 WPI	-	+ (1/2)	+ (1/16)	-	+ (1/8)	+ (1/64)
Vaccination Antigen	PBS	HV4297/7 R/95	HV 1976	PBS	HV4297/7 R/95	HV 1976

Table 4 SN results for tortoises immunized with HV4295/7R/95 and HV1976, IN (4a) and IM (4b).

In table 4a and 4b are reported the SN titers obtained for the plasma samples collected from the tortoises in the immunization study from time 0 to 36 and 34 weeks after immunization (IN and IM inoculation respectively). On the left side of each tables are reported the results from the SN test when the tortoise plasma samples were tested against HV4295/7R/95 used as antigen, while on the right are those obtained when tested against HV1976. On the bottom of each side of the table, the vaccination antigens are indicated. A total viral amount of 100TCID50 was used in the SN test.

Figure 1a. End point titration using HV 4295/7R/95 as antigen

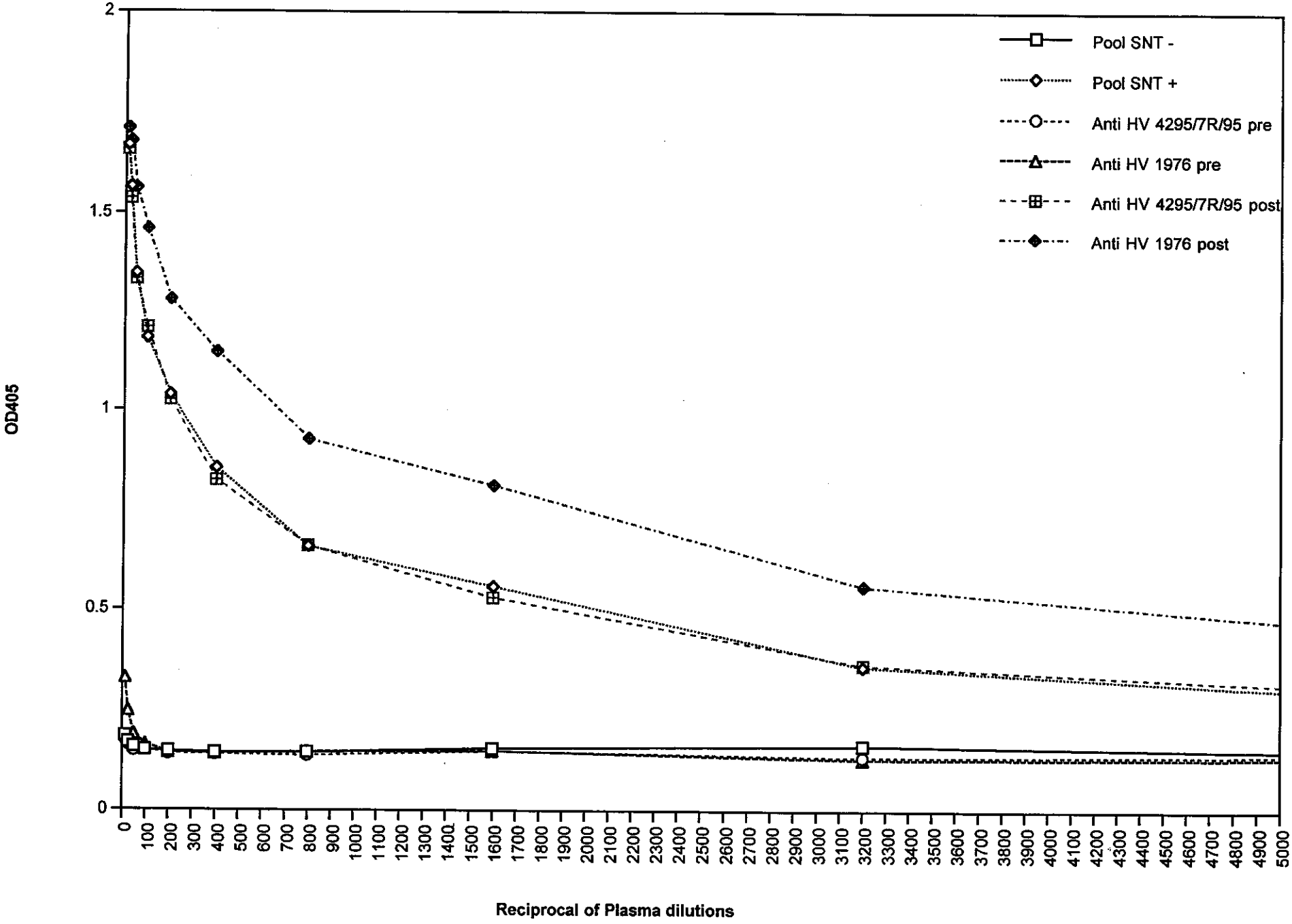

Figure 1a. End point titration using HV 4295/7R/95 as antigen

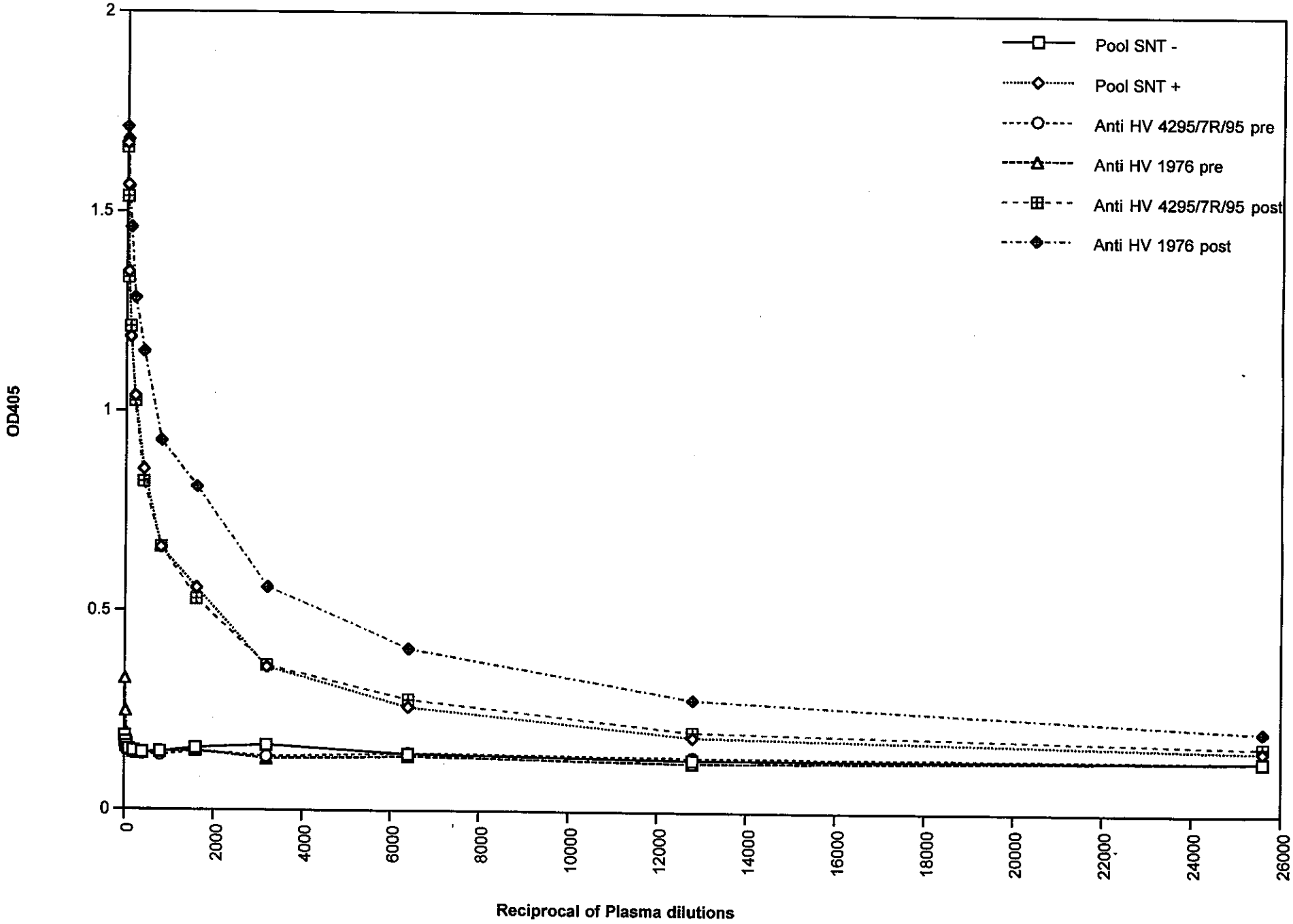

Figure 1b. End point titration using HV 1976 as antigen

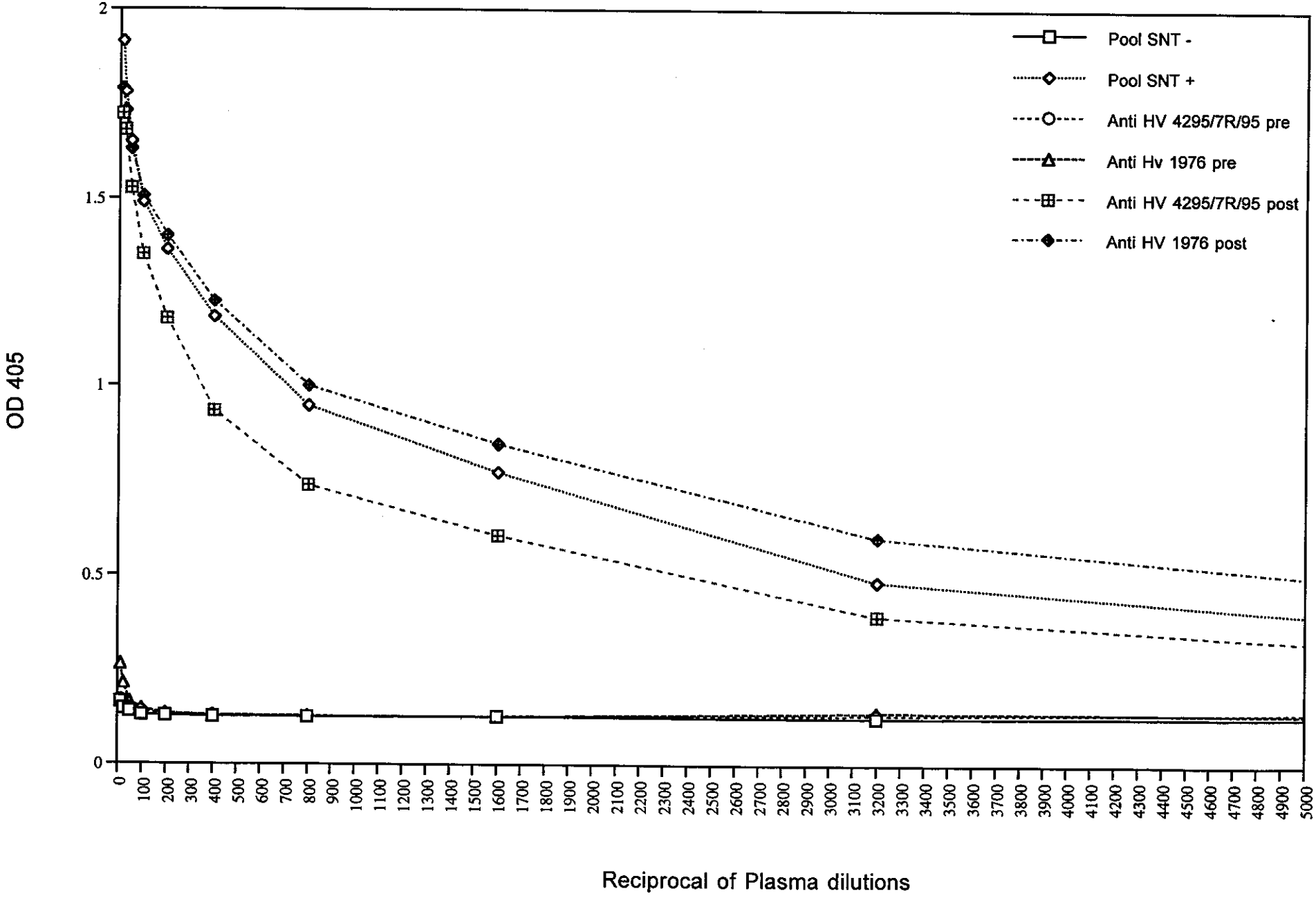

Figure 1b. End point titration using HV 1976 as antigen

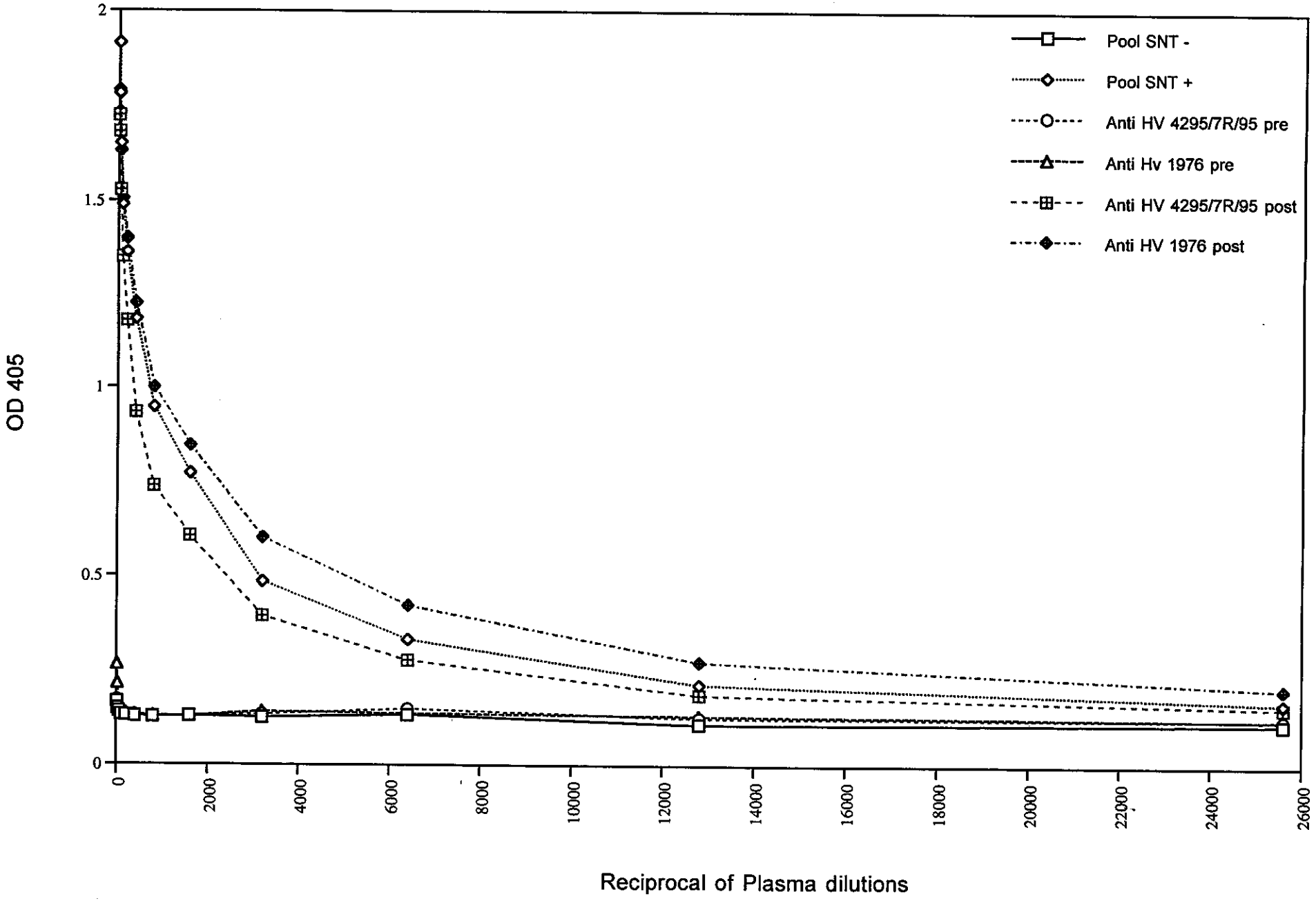

Figure 2

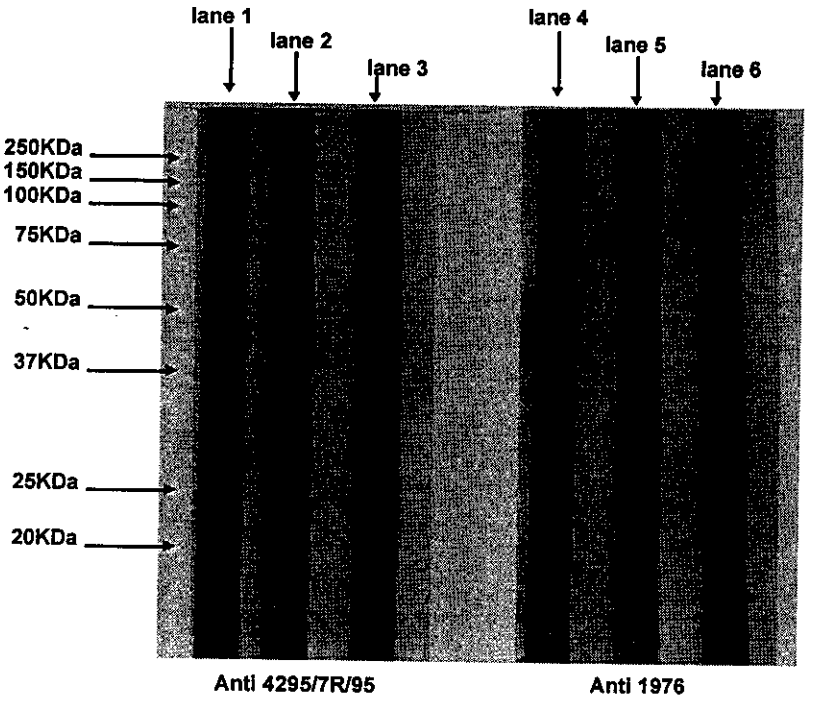

Figure 2 Comparision of the antigens of 4295/7R/95 and HV1976 using hyperimmune tortoise sera (antibodies reactions in lanes 1, 2 and 3 are obtained using tortoise sera raised against HV4295/7R/95; antibodies reactions in lanes 4, 5 and 6 are obtained using tortoise sera raised against HV 1976) (lane 1 = HV1976; lane 2 = HV4295/7R/95; lane 3 = TH-1 cell lysate; lane 4 = HV1976; lane 5 = HV4295/7R/95; lane 6 = TH-1 cell lysate)

Figure 2 shows the western blot results for the hyperimmune tortoise sera. Few antibody reactions are detectable, and a very faint band of approximately

20 KDa is observable in the infected cells when stained using anti-HV1976 polyclonal tortoise sera. An antibody reaction with a similar molecular weight is detectable also in the HV4295/7R/95 infected cells, using anti-HV4295/7R/95 polyclonal tortoise sera. An antibody reaction of approximately the same size is detectable also in figure 3 in the infected cells. The arrows show the approximate molecular weight of the different antibody reactions

Figure 3.

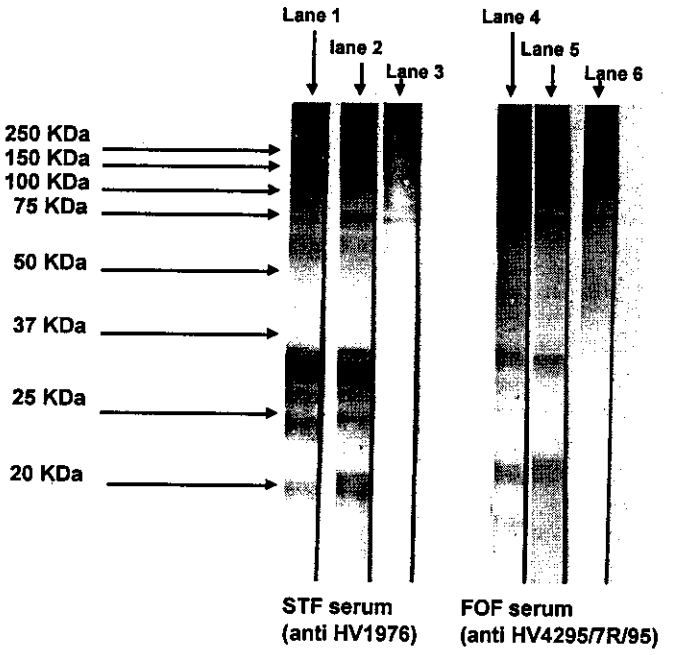

Figure 3 Comparision of the antigens of 4295/7R/95 and HV1976 using rabbit polyclonal antibodies raised against HV1976 (STF) (line 1, 2, and 3) and HV4295/7R/95 (FOF) (line 4, 5 and 6) (lane 1 = HV1976; lane 2 = HV4295/7R/95; lane 3 = TH-1 cell lysate; line 4 = HV1976; line 5 = HV4295/7R/95; line 6 = TH-1 cell lysate)

Several low molecular weight bands can be seen in lines 1, 2, 4 and 5and absent in lane 3 and 4 (uninfected TH-1 cells) (2a). FOF sera was raised against HV4295/7R/95, while STF was raised against HV1976. The arrows show the approximate molecular weight of the different bands

Figure 4a. Immunization data: Intranasal Inoculation (I.N.) -ELISA-

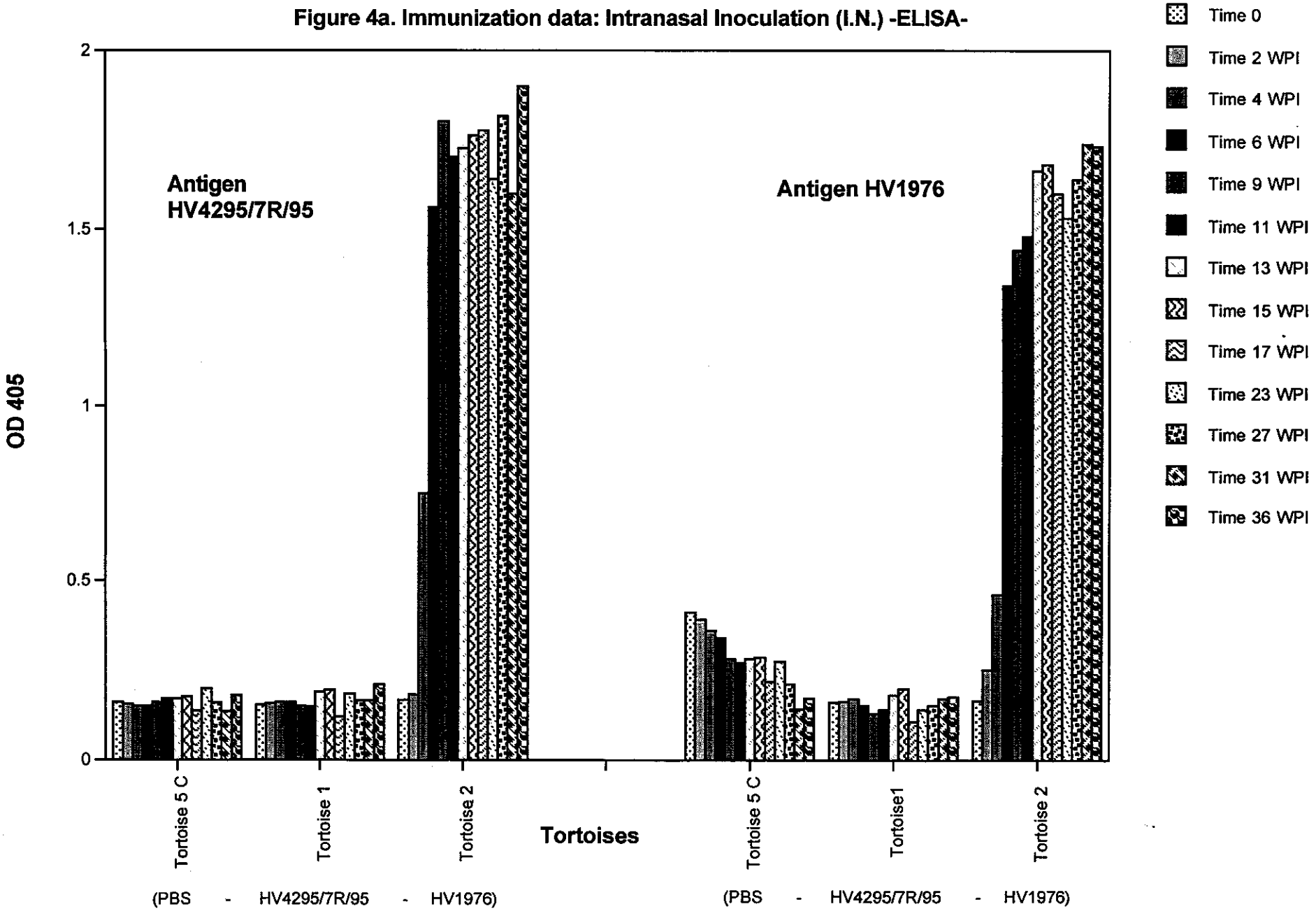

Figure 4b. Immunization data Intramuscular Inoculation (I.M.) -ELISA-